Thin-Layer (Planar) Chromatography

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Introduction

Synthetic dyes comprise a large group of organic, organic salt and organometallic compounds which number in the thousands. Most individual dyes are assigned colour index (CI) numbers which helps to identify structure and properties as well as identity where a series of names have been used by the manufacturers for the same dye. Pigments are also listed and can be either organic or inorganic in nature. The value of planar chromatography in the identification of dyes and separation of impurities and their quantification is the main subject of this article. Synthetic dyes will be split into nine groups, the first three being the major ones. For all these groups, the development of modern thin-layer chromatographic (TLC) methods will be compared where possible with older TLC procedures.

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Use of TLC for the Separation of Dyes

Synthetic dyes and pigments are used in a wide range of industries. They are the colours of printing inks, textile dyes, cosmetics, plastics, histological and cytological stains, and some are permitted as food and drink colorants. In some of these applications purity is important, in others it is the presence or absence of certain impurities or intermediates, and in still others it is the identification and uniformity that is important. In all these instances planar chromatography has proved to be the ideal solution with the separation power and spot/zone capacity necessary to resolve closely related dyes and intermediates. This has been made possible by the wide selection of stationary phases and the almost unlimited variations possible with mobile-phase mixtures, sometimes composed of quite aggressive solvents that would not be used in column liquid chromatography. The only limiting criteria for the solvent mixtures are adverse effects on the adsorbent binder, reaction with the bonded phases and high viscosity and surface tension of the solvents.

There are a number of other advantages to the use of TLC for the analysis of dyes compared with other chromatographic techniques. The most obvious is that dyes are easily visualized on a chromatographic layer by their colour. Often slight differences in hue are more clearly seen on the layer than in solution and hence are easily distinguishable. It is therefore rarely necessary to employ detection reagents unless the area of interest is dye intermediates which may lack the conjugation needed in their molecular structure to be coloured in visible light. Of course, there are a large number of dyes which either exhibit fluorescence quenching in short wavelength ultraviolet (UV) light (254 nm) or naturally fluoresce by excitation in long wavelength UV light (usually 366 nm). Where separated dyes on the chromatographic layer do fluoresce, the limit of sensitivity of detection is often in the low nanogram or high picogram level. In the commercial environment, as dye quality can vary from batch to batch and colour can be matched by using different dyes, the planar chromatographic technique allows the analysis of many samples against references or certified standards on the same layer under the same conditions in one development run. Hence the analysis time and the cost per sample are substantially lower when compared with liquid chromatography.

In most forms of chromatography, as in many spectroscopic analyses, the extraction of the dye from whatever matrix is being considered and the subsequent sample pretreatment are often time-consuming, but usually necessary as impure dyes notoriously contain many impurities that are easily and firmly retained on the chromatographic adsorbents. Liquid chromatographic columns and pre-columns quickly become 'poisoned' and then either have to be discarded or require extensive solvent clean-up before re-use. In TLC only minimal clean-up or extraction is required as most polar impurities will remain at or near the origin of application and more nonpolar ones will migrate with the solvent front (normal-phase separation). As the thin-layer plate is not re-used, the unseparated material at the origin or at the solvent front is of no consequence. However, the chromatograms obtained are a source of extensive data about the dye separation. Not only can the chromatographic tracks be scanned spectrodensitometrically, but individual UV/visible spectra of the separated chromatographic zones can be recorded, and a number of other spectroscopic techniques such as mass spectrometry (MS), nuclear magnetic resonance (NMR), Fourier transform infrared (FTIR) and Raman spectra can be applied.

For use in histology, many reference standards are available, as tested and approved by the Biological Stains Commission. Such dyes and stains are labelled accordingly, usually giving the dye content for the batch as numbered. Most such dyes are single components, but where it is known to be composed of two or more components (e.g. methylene violet (3)), azure A or C (up to 6), methyl violet (4), aniline blue (3) and fast green FCF (3)), the dye content will be based on the named dye (usually the major component). The testing of dyes suitable for histology and cytology has always relied on a number of wet chemistry tests, UV/visible spectral analysis, TLC analysis and the actual performance as a stain. The wet chemistry tests include titanous chloride assay, sulfated ash and moisture content. The total dye content has been determined by titration with standard titanous chloride solution and measurement of absorbance (UV). High performance TLC (HPTLC) not only can be used to determine accurately the total dye content, but also the concentration of the individual components if, as so often happens, the sample is either a mixture of dyes or there are impurities present that are closely related structurally to the parent dye (e.g. determination of crystal violet (hexamethylpararosanilin) in methyl violet 2B, 3B, 6B or 10B (a mixture of various proportions of hexamethyl-, pentamethyl-, tetramethyl-, and trimethyl-pararosanilin)).

In most industrial uses of dyes (textiles, printing and plastics) and in forensic work, TLC is used for identification and on some occasions to quantify these results. In the commercial environment textile dyes are not normally marketed in the pure state. Both the end user and the manufacturer of the dyes are mainly interested in a dye of standard reproducible quality in their application rather than chemical purity. For this reason the TLC analysis of the dyes for textile, cosmetic or printing purposes presents different problems to that for staining purposes in histology. A commercial dye will often contain impurities composed of by-products of the dye synthesis and starting materials. Inorganic salts are usually present: these have been used to 'salt out' or precipitate the dye during manufacture or they may have been added as extenders, so that different batches have the same dyeing potential. Variations in colour hue are sometimes adjusted by the addition of another dye.

In histology and cytology, although the identity of the dye is important, the purity plays an important part. It has also been realized that other dye impurities need to be present to give a superior quality stain. Both Giemsa and Leishman's stain are classic examples of this. In food and drinks, TLC of dyes can also be a useful technique, as only a limited number of dyes are permitted for food use (**Tables 1–4**). These are indicated on the label of the food or drink product and can easily be identified by TLC. The presence and identification of nonpermitted food dyes is one of the strengths of TLC.

Table 1 Individual permitted food dyes (depending on country)

Permitted dye	*E number*	*CI number*
Amaranth	E123	16185
Brilliant black (black PN)	E151	28440
Brilliant blue FCF	E133	42090
Carmoisine (azorubine)	E122	14720
Erythosine	E127	45430
Ponceau 4R	E124	16255
Indigo carmine	E132	73015
Quinoline yellow	E104	47005
Red 2G	E128	18050
Sunset yellow FCF	E110	15985
Tartrazine	E102	19140
Yellow 2G (food yellow 5)	E107	

Many of the early planar chromatography methods for dye analysis in the 1960s used paper or cellulose thin layers. Later, more methods, some still currently used, were based on silica gel G and aluminium oxide thin layers. In more recent times, commercially available pre-coated silica gel and cellulose layers with better reproducibility have become the preferred adsorbents for dye separations. This has become particularly the case where quantitative determinations are required for dye content on HPTLC plates.

Table 2 Recommended solvents/solvent mixture for the separation of lipophilic solvent (fat) dyes on silica gel 60 pre-coated TLC plates

Solvent dye	*CI numbers*	*Solvents for separation*[a]
Sudan black B	26150	Dichloromethane
Fat red 7B	26050	Toluene
Sudan I	12055	Heptane–ethyl acetate (80 + 10 v/v)
Sudan II	12140	Cyclohexane–ethyl acetate (90 + 10 v/v)
Sudan III	26100	
Sudan IV	26105	
Butter yellow	11020	
Sudan red G	12150	
Indophenol blue	49700	
Diethyl yellow	11021	
Oil blue APS	61551	
Waxoline green G-FW	61565	
Waxoline red MP-FW	60505	
Waxoline yellow E	47000	
Oil red O	26125	

[a]These recommended solvents and solvent mixtures apply to all the solvent dyes.

Table 3 R_F values for a number of solvent (fat) dyes developed on silica gel 60 pre-coated plates

Solvent dye	R_F *values (dichloromethane)*	R_F *values (n-hexane–ethyl acetate (90 + 10 v/v))*
Diethyl yellow	0.72	
Oil blue APS	0.34	
Waxoline green G-FW	0.64	
Waxoline red MP-FW	0.54	
Waxoline yellow E	0.12	
Sudan black B	0.10 (f), 0.18 (f), 0.22 (m), 0.61 (m), 0.85 (f)	
Waxoline blue	0.43 (m), 0.13, 0.79	
Sudan I		0.68
Sudan II		0.2
Sudan III		0.56
Sudan IV		0.56
Sudan orange G		0.14
Sudan R		0.18

f, Faint zone; m, main zone.

For the purposes of planar chromatography, separations of synthetic dyes can be split into a number of groups depending on the ionic nature and molecular structure of the dye. These are:

1. Solvent (fat) dyes.
2. Basic dyes.
3. Acid dyes.
4. Reactive dyes.
5. Disperse dyes.
6. Metal complex dyes.
7. Direct dyes.
8. Organic pigments.
9. Food colorants.

As mixtures of dyes generally fall into these respective groups and it is rare, if ever, that a dye from one of the major structural groups is found mixed with another, the TLC will be examined in detail for each group in turn. This is important as the polarity of solvents employed for the TLC separations of dyes within each group is similar and notably different from those used for other groups.

Solvent (Fat) Dyes

As their name suggests, solvent dyes are dyes soluble in nonpolar solvents (mostly water-immiscible), and are used to colour mineral oils, waxes, fats and plastics. They are lipophilic in nature and do not form organic salts. Structurally the dyes are characterized by the azo, quinone-imine (indophenol dyes), or anthraquinone (waxoline dyes) groups they contain. By far the largest group is the azo dyes. These are mainly monoazo (sudan dyes) and a few disazo dyes (e.g. Sudan red B (CI 26110), oil red O (CI 26125; **Figure 1**).

Although TLC separations of solvent dyes have been reported on alumina layers, silica gel is by far the preferred adsorbent. Separation of solvent dyes

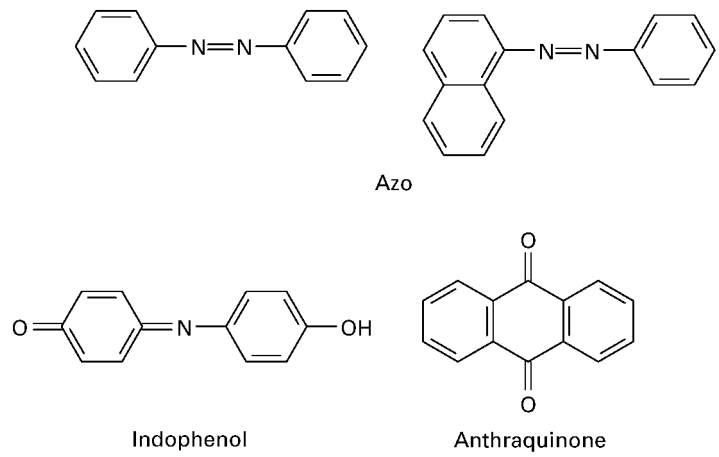

Figure 1 Solvent dyes. Structures of characteristic groups.

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Table 4 R_F values for Sudan III and IV on silica gel 60 RP8 HPTLC pre-coated plates. Plate developed in a saturated chamber

Solvent dye	R_F *value*	*Developing solvent*
Sudan III	0.32	Methanol–acetic acid–water
Sudan IV	0.22	(90 + 5 + 5 v/v)

on standardized silica gel G layers was reported in the early 1960s by Stahl. This was one of the first recorded separations on silica gel and described the resolution of butter yellow (CI 11020), Sudan R (CI 12150) and indophenol (CI 49700) using a mobile phase of pure benzene. In the years that followed, a variety of similar-polarity solvents were reported as suitable with silica gel G. Chloroform and benzene and mixtures of petroleum spirits, and single aliphatic hydrocarbons with ethyl acetate, diethyl ether, acetone and methanol were used to separate butter yellow, Sudan orange R (CI 12055), Sudan III (CI 26100) and Sudan red BB (CI 26105). The anthraquinone series of dyes have been separated with toluene–cyclohexane (50 + 50% v/v) on silica gel G. This readily resolves solvent blue 36, waxoline purple A (CI 60725) and waxoline green G (CI 61565). Dimethylaminoazobenzene and related dyes are separated using the solvent chloroform–methanol (95 + 5% v/v) on the same stationary phase.

Most solvent dyes will readily migrate and separate from each other on commercially pre-coated silica gel 60 TLC layers using the same or similar polarity solvents to those used on silica gel G. However, the separated chromatographic zones on pre-coated plates are much sharper, noticeably less diffuse and misshapen, leading to good overall resolution (**Figures 2** and **3**). This should be expected as the silica gel will have a much narrower particle size distribution over the particle size range, and a mean particle size (10–12 μm) less than that of a 'home-made' plate.

The resolution can be further improved for Sudan dyes by using a reversed-phase adsorbent. In fact, the resolution is so good that results can be quantified on an HPTLC silica gel 60 RP8 plate (Merck) by scanning the developed plate at 540 nm (**Figure 4**). In this example, samples were applied as spots of 100 nL with a manual nanoapplicator (CAMAG). The relative standard deviation was 4.5%.

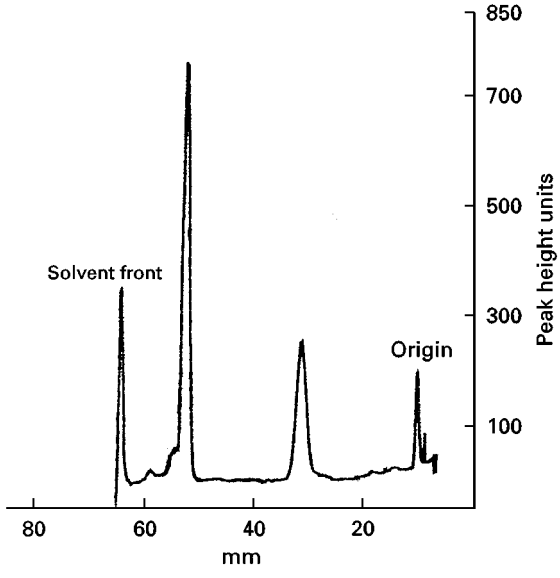

Figure 2 Separation of two main components of Sudan black B on silica gel 60 pre-coated TLC plates. Developed in a saturated tank with dichloromethane as solvent. Scanned with a spectrodensitometer at 540 nm.

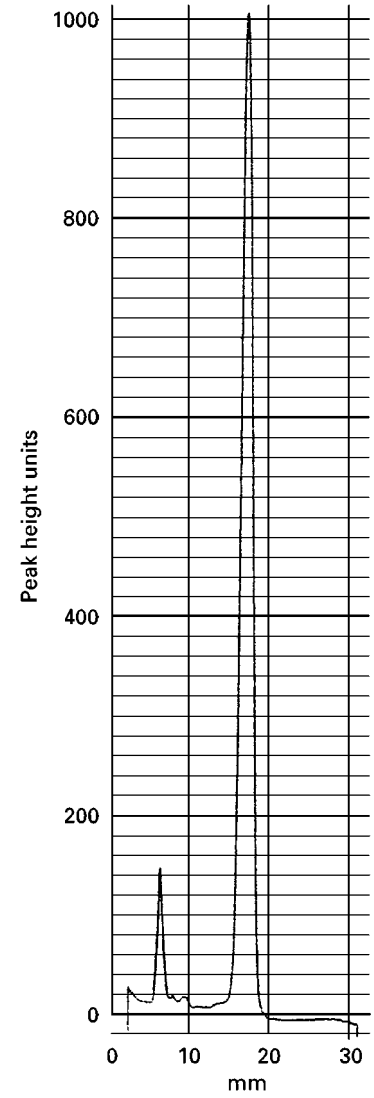

Figure 3 Separation of fat red 7B from one main impurity on silica gel 60 pre-coated TLC plates.

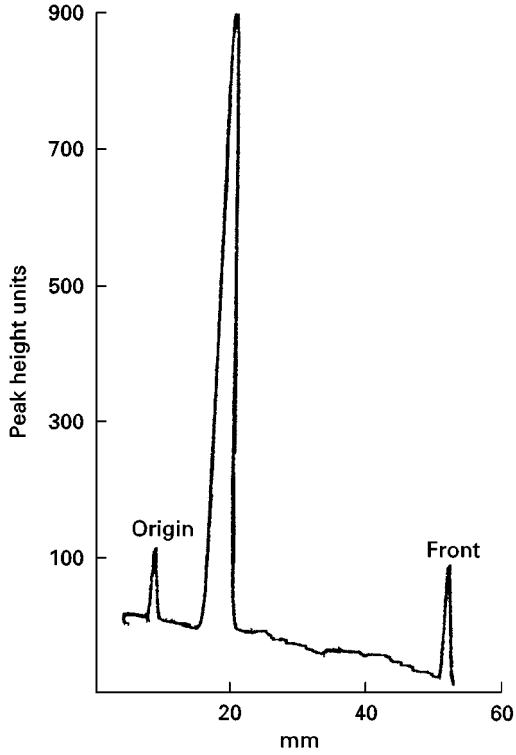

Figure 4 Pure Sudan IV resolved on silica gel 60 TLC plates using *n*-hexane–ethyl acetate (90 + 10 v/v) as mobile phase in a saturated tank.

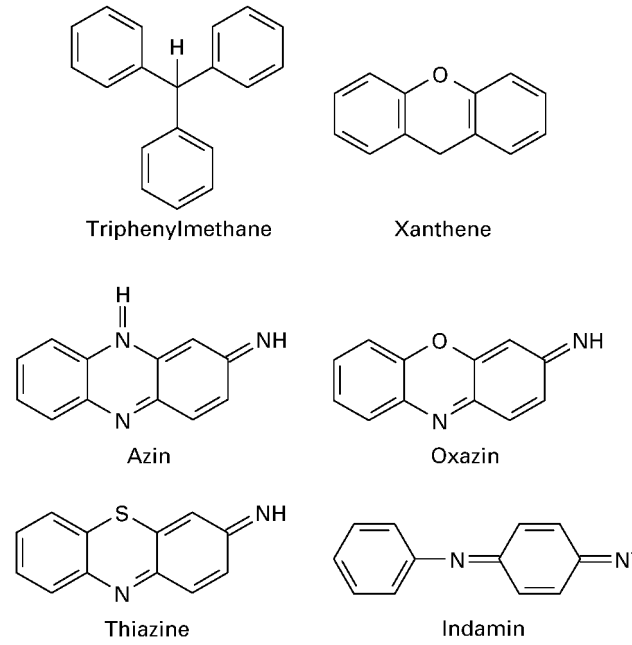

Figure 5 Acid and basic dyes. Structures of characteristic groups.

Basic Dyes

Basic dyes form one of the larger areas of interest when it comes to TLC. As their name suggests, they are bases which can form salts which are soluble in water. Their applications vary widely in industry, including colouring paper, some textiles and plastics, and are used as the basis of many biological stains.

Basic dyes include the following structural groups: triarylmethanes (mostly triphenylmethanes), quinoneimines, azo, acridine and xanthene compounds (**Figure 5**). These can be further split: the triarylmethanes into amino and hydroxy derivatives and the quinoneimines into indamins, azins, thiazins and oxazines. **Table 5** shows some of the commonly named dyes which fit into these groups.

As with solvent dyes, much of the early separation work was performed on silica gel G. **Table 6** lists a number of solvent mixtures which have proved useful in the separation of the general classes of basic dyes. With the advent of commercial TLC and HPTLC pre-coated silica gel 60 and bonded phases, the separation methods have improved, allowing resolution of dyes with closely related structures, and the determination of concentrations of individual dyes by *in situ* scanning using a spectrodensitometer (**Table 7**). Hence thiazin dyes are now easily resolved, including

Table 5 Commonly named basic dyes according to their structural groups

Basic dye groups	*Commonly named dyes*
Triaminotriarylmethane	Pararosanilin
	New fuchsin
	Rosanilin
Diaminotriarylmethane	Malachite green
	Brilliant green
	Victoria blue
Monoaminotriarylmethane	Methyl violet
	Crystal violet
	Ethyl violet
Acridine	Acridine red
	Acridine orange
Xanthene	Pyronin B
	Pyronin G
	Rhodamine B
	Rhodamine 6G
Azin	Safranine O
Oxazine	Brilliant cresyl blue
	Orcein
	Resazurin
	Gallocyanine
	Cresyl fast violet
	Nile blue
	Cellestine blue
Thiazin	Azure A, B, C
	Methylene blue
	Toluidine blue
	New methylene blue
	Thionine
	Methylene violet
Azo	Janus green
	Chrysoidin
	Bismarck brown R and Y

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Table 6 Solvent systems recommended for the separation of basic dyes on silica gel 60 pre-coated TLC plates and sheets

Basic dyes	*Solvent mixtures*
Malachite green, methyl violet	Butan-1-ol–ethanol–water (90 + 10 + 10 v/v)
Basic fuchsin, rhodamine B, rhodamine 6G	Butan-1-ol–acetic acid–water (40 + 10 + 50 v/v)
Methylene blue, victoria blue B, crystal violet, malachite green, rhodamine B	Butanone–acetic acid–propan-2-ol (40 + 40+ 20 v/v)
Pyronine G, acridine red, rhodamine B	Propan-1-ol–formic acid (80 + 20 v/v)
Methylene blue, malachite green	Ethyl acetate–acetic acid–water (30 + 10 + 20 v/v)
Crystal violet, methylene blue, malachite green, basic fuchsin	Butan-1-ol–acetic acid–water (20 + 5 + 10 v/v)

thionine, azure A, B, C and methylene blue on HPTLC silica gel 60 using a mobile phase of butan-1-ol–butanone–acetic acid–water (30 + 20 + 10 + 10 v/v). Methylene blue can easily be distinguished from new methylene blue even though their visible/UV spectra are almost identical (**Figure 6**).

Many of these dyes are the basis of the biological Romanowski stains (Giemsa, Leishman, Wright, Jenner and May-Grunwald). An assessment of the dye quality of the stain and its individual dye components can be made by the above chromatographic methods. Usually the 'ripened' or oxidized versions of these stains produce the better histological staining properties. Oxidation produces more azures and thionine, all of which can be determined, if required, by HPTLC procedures. The complete resolution of methylene blue and its oxidation products including azures and thionine presents a difficult problem even for HPTLC as these dyes are all closely structurally related. A typical example of such a separation is shown in **Figure 7** (separation of the blue dye components of Wright's stain) on a normal-phase pre-coated silica gel 60 plate (Merck).

For most basic dyes, TLC and HPTLC silica gel 60 layers normally give sufficient resolution and have proved in some cases to be suitable even for the separation of dyes from closely related impurities (**Figure 8**). In special cases, either reversed-phase layers or other bonded layers have solved particularly difficult separation problems. One of these has been the separation of pararosanilin from rosanilin, new fuchsin and magenta II. Pure pararosanilin is used in the preparation of Feulgen and Schiff's reagent for aldehyde and ketone detection and in biological staining. All four compounds are triaminotriphenylmethane dyes, differing consecutively by one methyl group. Although silica gel 60 RP8 HPTLC layers are able to resolve just the four components, tailing problems make it difficult to scan the zones quantitatively. However, by introducing the ion-pairing reagent

Table 7 Solvent systems for individual basic dyes on silica gel 60 TLC and HPTLC pre-coated plates. R_F values obtained and recommended scanning wavelength for detection

Basic dye	*Solvent mixture*	R_F *value*	*Detection wavelength*
Brilliant cresyl blue	Ethyl acetate–methanol–ammonia (0.88)–water (35 + 11 + 5 + 5 v/v)	0.56	605 nm
Crystal violet	Butan-1-ol–acetic acid–water (50 + 5 + 10 v/v)	0.39	620 nm
Ethyl violet		0.58	585 nm
Acridine orange	Ethyl acetate–methanol–ammonia (0.88)–water (33 + 11 + 5 + 5 v/v)	0.64	360 nm (fluorescence)
Azure B	Butan-1-ol–acetic acid–water (50 + 5 + 10 v/v)	0.12 (m), 0.19 (m), 0.29 (m), 0.33, 0.40	620 nm
Brilliant green		0.46	620 nm
Malachite green		0.36	620 nm
Methyl violet 6B		0.39 (m), 0.46, 0.51	620 nm
Methylene blue	Butan-1-ol–acetic acid–water (50 + 10 + 20 v/v)	0.29 (m), 0.34	620 nm
New methylene blue	Butan-1-ol–acetic acid–water (50 + 5 + 10 v/v)	0.32	610 nm
Nile blue		0.48	620 nm
Orcein	Ethyl acetate–methanol–ammonia (0.88)–water (35 + 11 + 5 + 5 v/v)	0.44 (m), origin, 0.19, 0.26, 0.37, 0.39, 0.41, 0.42, 0.47, 0.48, 0.49, 0.54	Visual
Pyronin G		0.27	360 nm (fluorescence)
Rhodamine B	Butan-1-ol–acetic acid–water (50 + 5 + 10 v/v)	0.48	540 nm
Safranine O		0.45 (m), 0.53	540 nm
Thionine	Butan-1-ol–acetic acid–water (50 + 10 + 20 v/v)	0.50	620 nm

m, Main zone.

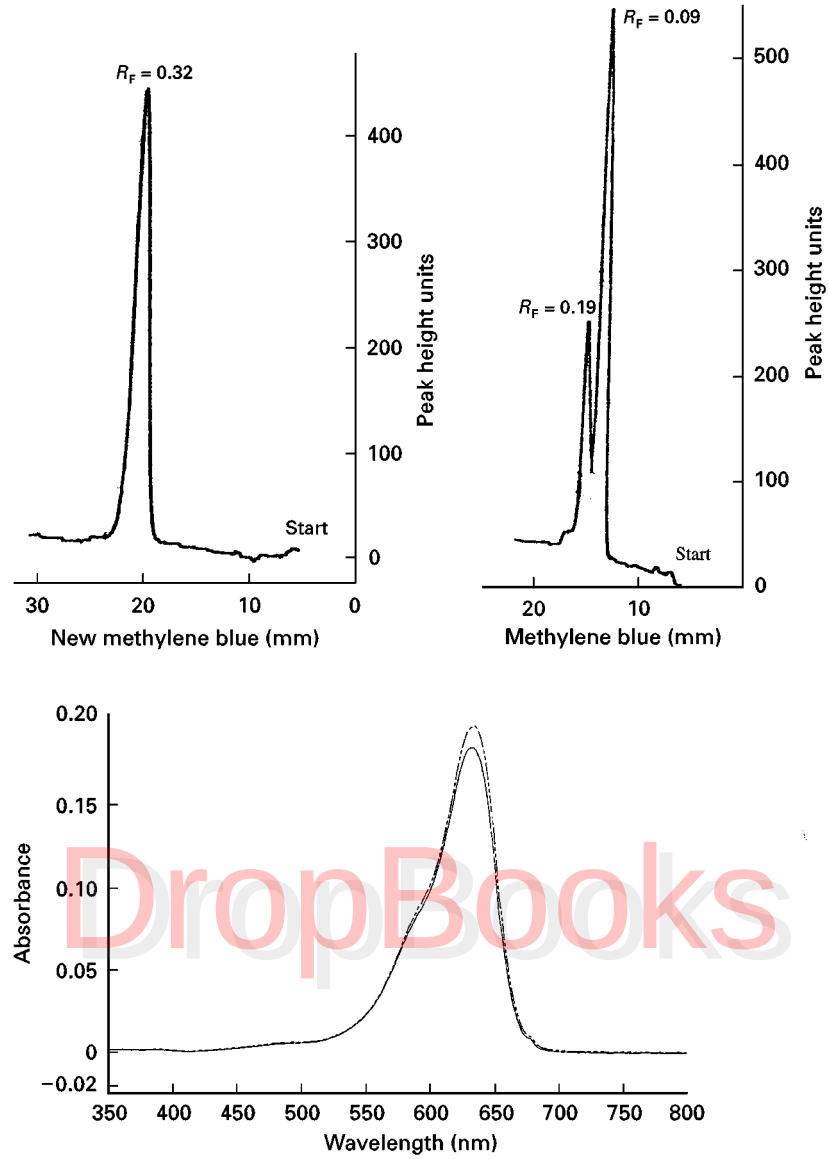

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Figure 6 Comparison of methylene blue separation with new methylene blue. Both separated on silica gel 60 pre-coated TLC plates with butan-1-ol–acetic acid–water (50 + 5 + 10 v/v) as mobile phase. Overlaid spectra also shown.

pentanesulfonic acid sodium salt (2%) into the developing solvent mixture (methanol–water–formic acid: 75 + 20 + 5 v/v), the effect of tailing is dramatically reduced, allowing quantitative determination of all four dyes. Another difficult separation problem arises with Bismarck brown Y and R. Resolving the dyes from their impurities is not easily achieved. In this instance silica gel 60 NH_2-bonded HPTLC layers have proved successful (**Figure 9**).

Acid Dyes

Like basic dyes, acid dyes are used widely throughout industry. They are the basis of inks and biological stains and are used for colouring wool, polymer fibres, leather and paper. They are mostly sulfonic and carboxylic acid compounds, often prepared as their sodium salts to enable good water solubility. They can be split into a range of structural groups: azo, triarylmethane, xanthene, anthraquinone and quinone-imine. The separation of these groups on silica gel G and cellulose using a variety of solvent mixtures for development is given in a number of the older TLC books found in the Further Reading list.

Commercial pre-coated silica gel 60 TLC and HPTLC plates, where the adsorbent has a carefully controlled pore size and particle size, give improved resolution of acid dyes compared with that obtained

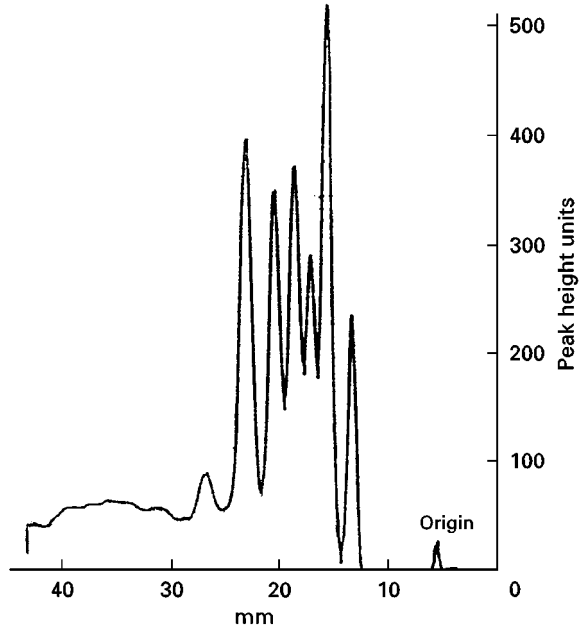

Figure 7 Separation of blue components of Wright's stain. Stationary phase is silica gel 60 pre-coated TLC plate. Mobile phase is ethyl acetate–methanol–ammonia solution (0.88)–water (35 + 11 + 5 + 5 v/v). Mixture of methylene blue, azures A, B and C, and thionine and other oxidation products.

for example with silica gel G. It has been possible to quantify results by spectrodensitometric scanning in a comparable way to basic dyes (**Figure 10**). Purity can be determined with a high degree of accuracy as is shown with the commercial samples of light green (**Figure 11**). Typically, five standards of certified dye are developed on the same plate alongside the samples. Peak measurements are made by reflectance and an x-power curve plotted against concentration. Unknowns are determined with about $\pm 1\%$ standard deviation. It is also possible to determine where rogue dyes have been used to try and replace the genuine dye. Examples of Congo red and brilliant cresyl blue are shown in **Plates 1** and **2**.

More recently, reversed-phase silica gel layers have proved useful for acid dyes where separation has not been possible on normal-phase silica gel. An example of this is eosin bluish shade, where reversed-phase plates have proved satisfactory, as shown in **Table 8**.

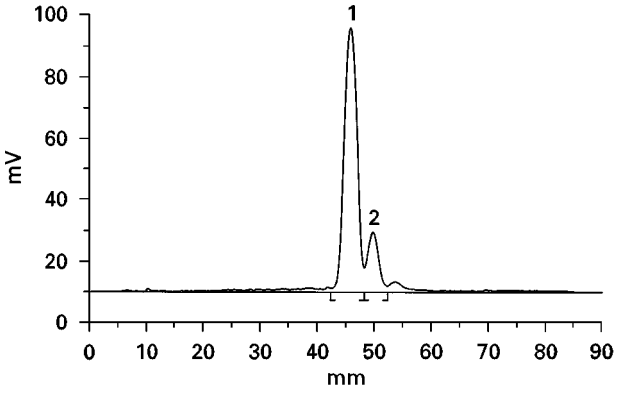

Figure 8 Separation of crystal violet from other closely related less methylated pararosanilins. Stationary phase: silica gel 60 pre-coated HPTLC plates. Mobile phase: butan-1-ol–acetic acid–water (50 + 5 + 10 v/v). Peak 1: hexamethylpararosanilin (crystal violet), peak 2: pentamethylpararosanilin.

Reactive Dyes

Reactive dyes attach themselves to cellulose fibres in wool and cotton. They are structurally defined into the groups azo, anthraquinone and phthalocyanine derivatives. One well-known commercial group is the procion dyes which can be separated on silica gel G with solvent mixtures – butan-1-ol–propan-1-ol–ethyl acetate–water (20 + 40 + 10 + 30 v/v) and dioxane–acetone (50 + 50 v/v).

Hydrolysis products of reactive dyes have also been examined by TLC on silica gel using the solvent mixture butan-1-ol–pyridine–water–ammonia (0.88 solution: 15 + 5 + 3 + 2 v/v), and with solvent mixture chloroform–methanol–acetone (18 + 3 + 2 v/v) with detection at 310 and 614 nm using a spectrodensitometer. The TLC of the reaction product and hydrolytic by-products of terminal ring isomers of reactive blue 2 (an anthraquinone dye) have been studied on silica gel using butan-1-ol–propan-2-ol–ethyl acetate–water (20 + 40 + 10 + 30) as developing solvent.

Disperse Dyes

Similar in structure to fat-soluble dyes, disperse dyes are mainly used to colour cellulose acetate and polymer fibres (principally polyester). They exhibit low solubility in water, but are soluble in organic solvents. Disperse dyes are types of nitrodiphenylamine, azo and anthraquinone dyes, but not those associated with either sulfonic or carboxylic acid groups. Commercially they belong to the Celliton group of dyes which can be separated by TLC on silica gel G adsorbent using chloroform–methanol (95 + 5 v/v) as mobile phase. Better separations of amino derivatives of anthraquinones have been obtained using chloroform–acetone (90 + 10 v/v). Mixtures of chloroform–ethyl acetate (50 + 50 v/v) or various dilutions of toluene with acetone can be employed on pre-coated silica gel 60 layers. When analysis of the dyes is required in natural and synthetic polymers, dichloromethane, acetone or dimethylformamide can be used to extract them ready for application to the TLC plate.

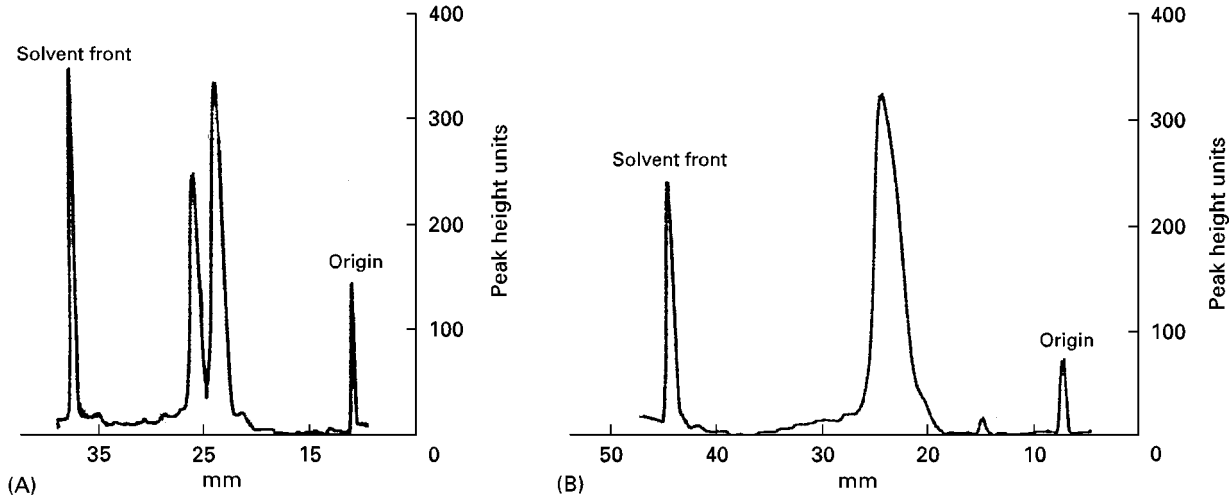

Figure 9 Separation of Bismarck brown Y and R on silica gel 60 NH_2 pre-coated HPTLC plates. Mobile phase: industrial methylated spirit. (A) The two components of Bismarck brown Y; (B) Bismarck brown R.

Metal Complex Dyes

These are acid dyes, mostly belonging to the azo group. They can be divided into two distinct types: 1 : 1 and 1 : 2 complexes.

1 : 1 Complexes

These are water-soluble dyes of sulfonic acid type which can be separated by TLC on silica gel using the solvent systems described under the section on acid dyes. For the most part they are chromium complex dyes.

1 : 2 Complexes

These are water-insoluble, but solvent-soluble dyes. They are metal complexes of chromium and cobalt. The best adsorbent for separation on thin layers is polyamide using a solvent mixture of methanol–water–ammonia (0.88 solution: 80 + 16 + 4 v/v).

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Direct Dyes

Direct dyes are used for dyeing cotton fibres and leather. They are mainly polyazo dyes which can be

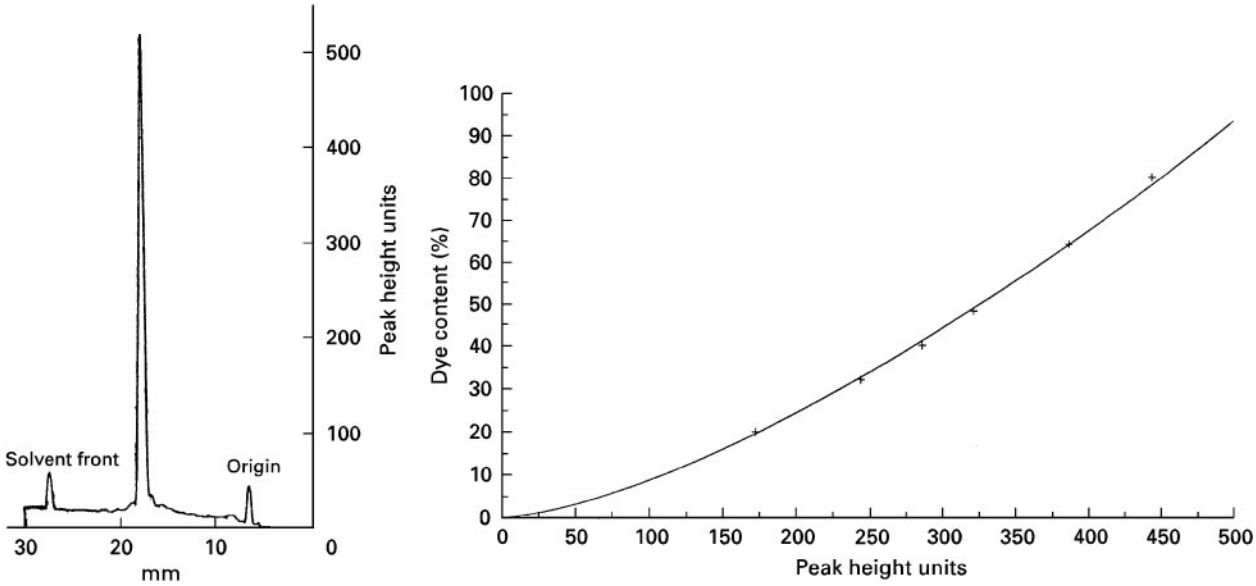
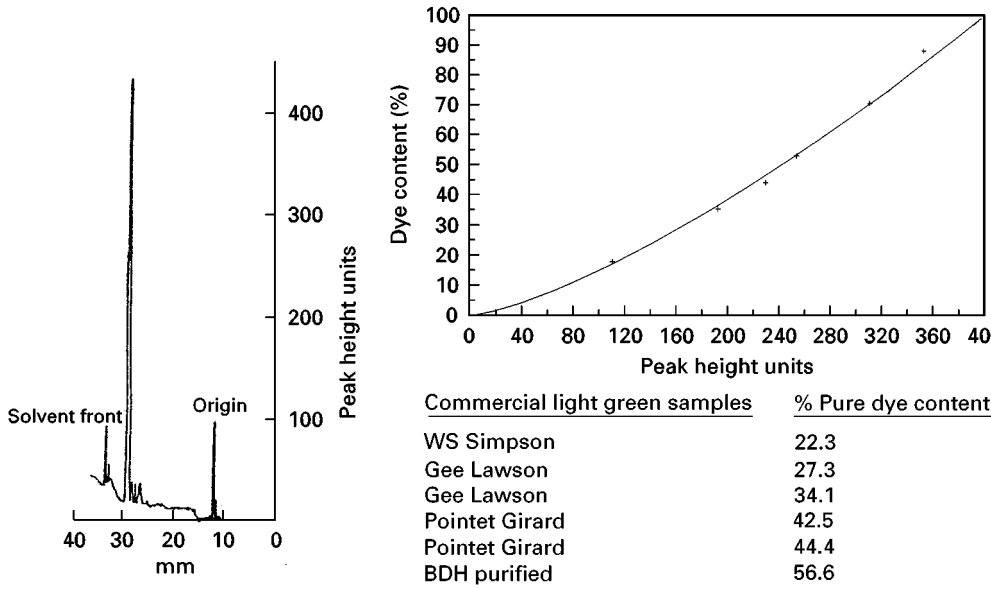


Figure 10 Quantification of Congo red dye. Single component dye. Typical x-power fit for reflectance data against concentration for a batch of certified dye. Stationary phase: silica gel 60 pre-coated HPTLC plates. Mobile phase: ethyl acetate–methanol–ammonia solution (0.88)–water (35 + 11 + 5 + 5 v/v).



Commercial light green samples	% Pure dye content
WS Simpson	22.3
Gee Lawson	27.3
Gee Lawson	34.1
Pointet Girard	42.5
Pointet Girard	44.4
BDH purified	56.6

Figure 11 Quantification of light green on silica gel 60 pre-coated HPTLC plates. Six standards applied as spots of nL loadings using a certified light green dye. The pure dye content of a number of commercial light green batches are listed as determined using this chromatographic procedure.

well separated on silica gel layers. Some, like Congo red, Evan's blue and trypan blue, have already been discussed under the acid dye section, but others can be resolved using the same solvent mixtures as recommended for the above dyes in **Table 9**. Slight modifications to the ratio of the solvents can often further optimize the solvent mixture for the specific dye.

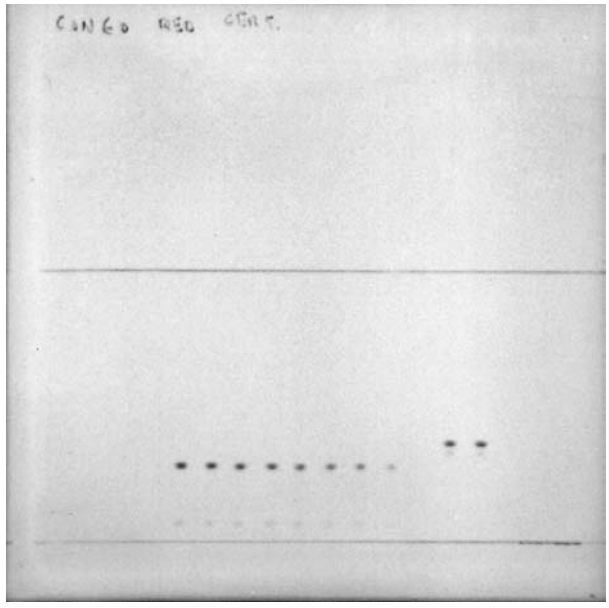


Figure 12 (See Colour Plate 78). Set of certified Congo red standards developed on a silica gel 60 HPTLC layer. Mobile phase: ethyl acetate–methanol–ammonia solution (0.88)–water (35 + 11 + 5 + 5 v/v). A minor impurity is visible ar R_F value 0.07. The two dye substances alongside these are of a dye that was claimed to be Congo red.

Organic Pigments

These are the colours of artist's paints which are also used for pigmenting rubbers and plastics. Included

Figure 13 (See Colour Plate 79). Separation of brilliant cresyl blue on silica gel 60 HPTLC plate. Mobile phase: ethyl acetate–methanol–ammonia solution (0.88)–water (35 + 11 + 5 + 5 v/v), saturated tank. Six standards applied at increasing concentration (from 50 ng) on the right side of the plate. The six samples developed on the left, all of equal concentration, were claimed to be brilliant cresyl blue, but in fact were a mixture of toluidine blue and Nile blue sulfate.

Table 8 Commonly named acid dyes according to their structural groups

Acid dye groups	*Commonly named dyes*	*Colour index numbers*
Azo	Methyl red	13020
	Methyl orange	13025
	Tropaeolin O	14270
	Orange II	15510
	Ponceau 2R	16150
	Orange G	16230
	Chromotrope 2R	16570
	Tartrazine	19140
	Congo red	22120
	Trypan blue	23850
	Evans blue	23860
	Ponceau S	27195
Triarylmethane	Acid fuchsin	42685
	Fast green FCF	42053
	Light green SF	42095
	Alkali blue 5B	42750
	Aniline blue	42755
	Xylene cyanol FF	43535
	Xylenol orange	
	Sulfonphthaleins	
Xanthene	Fluorescein	45350
	Eosin Y	45380
	Phloxine	45405
	Erythrosine B	45430
	Rose bengal	45440
Quinone-imine	Azocarmine G	50085
	Azocarmine B	50090
Anthraquinone	Alizarin red S	58005
	Indigo carmine	73015

here are the phthalocyanine dyes along with some azo, vat and anthraquinone dyes. They are for the most part insoluble in both organic solvents and water. Hence chromatographic separations have proved difficult. However, some chromatographic work has been described. Some pigments will dissolve in hot dimethylformamide. They can then be applied as such to silica gel layers. Thorough drying is recommended before development with benzene or benzene modified with chloroform. The use of paper chromatography has also been described with propan-1-ol–ammonia (0.88 solution: 20 + 10 v/v) as mobile phase.

Food Colorants

Acid dyes are used as colorants in food and drink. In the past, the recommended stationary phase for food dye separations was either cellulose or modified celluloses (diethylaminoethyl (DEAE), PAB). However, as in other areas of modern TLC, silica gel 60 has taken over as the preferred stationary phase. The optimum chromatographic conditions have therefore to a large extent already been described in the acid dye section. However, for completeness the optimum resolution of many of these dyes is listed in **Table 10**. These dyes are all water-soluble and separate readily on thin layers of silica gel 60. As they are ionic in nature, it is important that either acid or base modifiers are used in the solvent mixture (e.g. acetic or formic acids or ammonia solution). This reduces 'tailing' or 'streaking' of chromatographic zones by either suppressing ionization or totally ionizing the components of the sample.

Commercial dyes for food use are often blends of permitted acid dyes, e.g. green food dye is usually a mixture of brilliant blue FCF (E133) and tartrazine (E102); red food dye can be a mixture of red 2G (E128) and tartrazine (E102). The TLC methods must therefore have the ability not only to separate impurities, but also to resolve the parent dyes.

Most food dyes are prepared for use in a glycerine base. Although little pre-chromatographic preparation of samples is required, it is important where this base is used to solubilize these dyes further with water–methanol (50 : 50 v/v) so that when the sample is applied to the plate, the sample solution will properly 'wet' the layer. Solutions can be filtered if required. As the dyes are so readily soluble in water, their extraction from the food matrix does not usually present a problem. For colours in drink, the sample normally only requires dilution with methanol before application as a spot or band to the chromatographic layer.

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Future prospects

Future prospects of dye analysis using TLC/HPTLC are likely to be closely linked with the use of video imaging techniques (charged coupled devices). Usually dye analysis involves many samples in a commercial environment. Hence a technique that has the ability to take a video image of an entire developed plate which could contain many chromatograms, and has the software capabilities to determine concentrations with some degree of accuracy from the image density of the chromatographic zones, has a real benefit to the analyst. Hard-copy pictures of the chromatograms can be produced for entry into analytical reports. The speed of such analysis will undoubtedly improve as the speed of computation becomes ever faster and further software improvements are made.

The identification and possible quantification of minor dye impurities will also become more feasible as hyphenated techniques such as FTIR, MS and Raman spectroscopy are linked to HPTLC and become more widely used. Manufacturers of pre-coated plates are now commercially producing smaller particle size adsorbents (3–5 μm), some based on spheri-

Table 9 Solvent systems for individual acid dyes on silica gel 60 TLC and HPTLC pre-coated plates. R_F values obtained and recommended scanning wavelength for detection

Acid dye	*Solvent mixture*	R_F *values*	*Detection wavelength*
Alizarin red S	Butan-1-ol–acetic acid–water (50 + 10 + 20 v/v)	0.29 (m), 0.22, 0.46	Visual
Aniline blue		0.22 (m), 0.39 (m), 0.08, 0.27, 0.32, 0.49, 0.64	620 nm
Fast green FCF		0.31	620 nm
Light green SF		0.19	620 nm
Orange G		0.28	470 nm
Alkali blue 5B, 6B	Ethyl acetate–methanol–ammonia (0.88)–water (35 + 11 + 5 + 5 v/v)	0.28, 0.35, 0.41, 0.47, 0.52, 0.55, 0.87, 0.91	540 nm
Congo red		0.47	470 nm
Crystal ponceau	Butan-1-ol–IMS–water–ammonia (0.88) (50 + 25 + 25 + 10 v/v)	0.45	470 nm
Ponceau S		0.37	470 nm
Indigo carmine		0.53	620 nm
Acid fuchsin		0.46 (m), 0.33, 0.42	540 nm
Eosin Y	Ethyl acetate–methanol–ammonia (0.88)–water (33 + 11 + 5 + 5 v/v)	0.42 (m), 0.38	470 nm
Erythrosin B		0.43	540 nm
Naphthol yellow S		0.33 (m), 0.44 (f)	400 nm
Evans blue	Ethyl acetate–methanol–ammonia (0.88)–water (35 + 11 + 5 + 6 v/v)	0.16 (m), 0.42 (f)	620 nm
Fast garnet GBC salt	Butan-1-ol–acetic acid–water (50 + 5 + 5 v/v)	0.82 (m), 0.48 (f)	400 nm
Trypan blue	Ethyl acetate–methanol–ammonia (0.88)–water (35 + 11 + 5 + 6 v/v)	0.11 (m), 0.32, 0.42	620 nm
Azocarmine G	Ethyl acetate–methanol–ammonia (0.88)–water (35 + 11 + 6 + 4 v/v)	0.28	540 nm
Phloxine	Ethyl acetate–methanol–ammonia (0.88)–water (33 + 11 + 2 + 2 v/v)	0.38	540 nm

m, Main zone; f, faint zone.

cal shaped particles. These will have the advantage of better flow characteristics for the migrating solvent, although slower flow rates and, most importantly for dye impurity analysis, improvement in sensitivity. The smaller average particle size and spherical shape will mean that such minor components on the layer will be more concentrated with less diffusion than that presently seen on an HPTLC plate.

Table 10 Solvent systems and R_F values for food dyes separated on silica gel 60 pre-coated TLC plates

Food dye	*Solvent system*	R_F *value*
Erythrosin	Ethyl acetate–methanol–ammonia (0.88 solution)–water (33 + 11 + 5 + 5 v/v)	0.43
Sunset yellow		0.33
Tartrazine		0.11
Indigo carmine	Butan-1-ol–IMS–water–ammonia (0.88 solution: 50 + 25 + 25 + 10 v/v)	0.53
Red 2G	Ethyl acetate–methanol–water–ammonia (0.88 solution: 30 + 15 + 5 + 10 v/v)	
Brilliant blue FCF		

IMS, industrial methylated spirits.

See Colour Plates 77, 78, 79.

See also: **II / Chromatography: Thin-Layer (Planar):** Densetometry and Image Analysis; Mass Spectrometry. **III / Thin-Layer Chromatography-Vibration Spectroscopy. Dyes:** High-Speed Countercurrent Chromatography; Liquid Chromatography.

Further Reading

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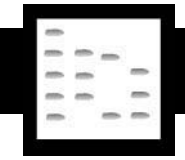
ECDYSTEROIDS: CHROMATOGRAPHY



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Introduction

Ecdysteroids are present both in animals (mainly Arthropods) and plants and comprise about 300 different molecules related to ecdysone (**Figure 1**). Structural variation in the number of carbons on the side-chain and of substituents at various positions (**Table 1**) results in the presence of compounds displaying very different polarities. Most available chromatographic techniques have been applied to the isolation and analysis of ecdysteroids. Paper chromatography is now obsolete and no longer used. Gas chromatography (GC) is of limited use, as the derivatization procedures necessary to make volatile derivatives require careful control. Currently, the most widely used techniques are high performance liquid chromatography (HPLC) and thin-layer chromatography (TLC), with the former providing the major analytical methods.

Liquid–Liquid Partitions

The simplest separation method concerns partitioning between two non-miscible solvents, and it is currently used for clean-up of biological samples. On this basis, several procedures have been designed, which allow the preparation of almost pure compounds in the gram scale.

Solvent Partitioning

Partition between n-butanol and water can be used to remove polar contaminants, whereas partition between aqueous methanol and hexane removes non-polar materials. Lipids can also be removed from aqueous extracts with hexane–methanol (7 : 3, v/v), light petroleum or n-propanol–hexane (3 : 1, v/v). The nature of the contaminants to be removed and that of the ecdysteroids to be isolated govern the choice for a given partition system. The number of free -OH groups significantly affects partition coefficients (**Table 2**).

The combination of two successive partition steps allows the elimination of both polar and apolar contaminants. It is thus possible to combine (1) chloroform/water and (2) water/butanol. This results in a butanol-containing fraction that is significantly enriched. It is possible to select a narrower range of polarity by replacing chloroform with a more polar organic solvent, e.g. isobutyl acetate, that nevertheless allows ecdysteroids to remain in the water phase.

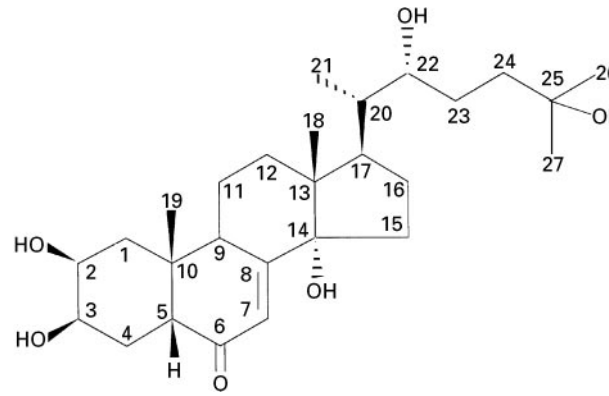

Figure 1 The structure of ecdysone, the first ecdysteroid isolated from *Bombyx mori* pupae.

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